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Formaldehyde Cross-Linking and Immunoprecipitation Demonstrate Developmental Changes in H1 Association with Transcriptionally Active Genes

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The in vivo association of histone H1 with specific genes in *Tetrahymena thermophila* was studied by using a simplified cross-linking and immunoprecipitation technique. Four genes were analyzed whose activities vary in three different developmental states (logarithmic growth, starvation, and conjugation). Hybridization of the immunoprecipitated DNA to cloned probes showed an inverse correlation between the level of immunoprecipitation with H1 antiserum and transcriptional activity. This represents the first demonstration of an alteration in histone H1-DNA interaction associated with developmental changes in transcriptional activity.

The lysine-rich histone H1 proteins are associated with the linker regions of chromatin and are proposed to serve as general repressors of gene expression by inducing the formation of a compact chromatin structure and by directly or indirectly preventing the binding of transcription factors (27, 36; reviewed in references 30, 31, and 33). Numerous correlative studies have demonstrated depletion of H1 in isolated active chromatin and enrichment in inactive chromatin (9, 10, 26); however, there is also ample evidence pointing to the presence of H1 in both transcriptionally active and inactive genes (12, 19, 23, 32). Experimentally, these studies involved methods subject to artifacts such as H1 redistribution, altered protein and DNA conformations, and perturbation of the linker regions by nucleases. It is well known that H1 is easily dissociable from chromatin, even under relatively mild conditions (see reference 31).

In the experiments described here, we have studied the association of H1 with chromatin in different states of transcriptional activity in *Tetrahymena thermophila*, a ciliated protozoan containing two different types of nuclei: a somatic macronucleus and a germinal micronucleus (16). During vegetative growth, transcription is restricted to the macronucleus. The sexual phase of the life cycle, conjugation, can be induced by mixing starved cells of different mating types (24). During conjugation, macronuclei degenerate while micronuclei undergo meiosis, fertilization, and division to produce nuclei that differentiate into new macro- and micronuclei. Although some micronuclear DNA sequences are transcribed during a brief period early in conjugation (21, 29), macronuclei are also the main source of gene activity in this stage. Even in the transcriptionally active macronucleus there are developmental changes in transcriptional activity (4), and it is at the level of transcript initiation that most genes are regulated in *T. thermophila* (28a). While about 50% of the single-copy DNA of macronuclei is expressed in both growing cells and starved cells that are competent to mate, a significant fraction of the expressed genes are unique to one or the other state (4). Also, genes have been cloned whose mRNAs accumulate in cells in a developmental stage-specific manner during conjugation (22). Thus, developmental activation of *Tetrahymena* genes can be viewed as a two-step process in which transcriptionally inert micronuclear genes become transcriptionally competent macronuclear genes that in turn become active in a developmental stage-specific manner.

Interestingly, the two nuclei have very different histones associated with the linker region of their chromatins. Macronuclei contain a very lysine rich protein with solubility properties, secondary modification, and amino acid composition of H1 but which lacks features of the central globular domain typical of metazoan H1s (38). Micronuclei contain four incompletely characterized linker-associated peptides that are distinct from H1 (1, 2). Thus, in *T. thermophila* as in other organisms, there is evidence suggesting that differences in linker histones are associated with marked differences in transcriptional competence. The question arises as to whether any changes in histone H1 accompany changes in transcriptional activity of specific genes in different developmental stages of the life cycle.

We have used an improved method of formaldehyde cross-linking in situ and immunoprecipitation to show that changes in the association of H1 accompany changes in transcriptional activity in different developmental stages.

Characterization of the cross-linking and immunoprecipitation technique. The technique of fractionating chromatin with specific antibodies to DNA-binding proteins has been used to study protein-DNA interactions (3, 14, 18, 28). The power of the method is increased by including a step to covalently bond the proteins to DNA, thus avoiding artifactual rearrangements. Solomon et al. (28) fixed *Drosophila* tissue culture cells directly with the reversible protein-protein and protein-DNA cross-linking agent formaldehyde (20), followed by immunoprecipitation with anti-H4 antibody to show that histone H4 remained associated with actively transcribed heat shock genes. We developed a modification of this formaldehyde fixation and immunoprecipitation technique by using crude serum and by removing a CsCl purification step and have adapted it for use on *Tetrahymena* macronuclear chromatin with H1 antiserum (10a). Briefly, the method involves in vivo fixation with 1% formaldehyde added directly to cultures of *T. thermophila* in logarithmic growth (17), starvation (17), or conjugation (50 to 70%

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pairing at 3 to 5 h) (22). After 3 min, the cells were washed at 4°C for 10 min in the following buffers: phosphate-buffered saline; 0.25% Triton X-100–10 mM EDTA–0.5 mM EGTA–10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), pH 6.5; and 200 mM NaCl–1 mM EDTA–0.5 mM EGTA–10 mM HEPES, pH 6.5. The cells were resuspended in 2% sodium dodecyl sulfate (SDS)–10 mM EDTA–50 mM Tris, pH 8.1, to a density of 0.5×10^7/ml, and the following protease inhibitors were added: leupeptin (1 μM), pepstatin (1 μM), and pheylmethylsulfonyl fluoride (1 mM). The chromatin was sheared to an average DNA fragment size of ~600 bp by 10 10-s bursts of sonication at 4°C. The sonic fluid was centrifuged (16,000 × *g*, 5 min) to pellet insoluble material and diluted 20-fold with 1% Triton X-100–2 mM EDTA–20 mM Tris, pH 8.1, and additional protease inhibitors.

Immunoprecipitation reaction mixtures contained chromatin equivalent to 5 μg of DNA and 200 μl of anti-H1 serum in a 10-ml final volume of 1% Triton X-100–0.1% SDS–2 mM EDTA–500 mM NaCl–20 mM Tris, pH 8.1, with fresh protease inhibitors. H1 antiserum was raised in rabbits against purified H1 protein as described previously (6). After an overnight reaction at 4°C, the immune complexes were precipitated with 6 serum volumes of protein A-Sepharose beads (Pharmacia; 1:1 slurry in 10 mM Tris–1 mM EDTA, pH 8). The complexes were eluted in 100 mM $NaHCO_3$–1% SDS after washing to remove nonspecifically bound DNA and chromatin. Following proteinase treatment and ethanol precipitation, the cross-links were reversed by heating to 65°C for 6 h and the DNA was purified by standard methods.

Optimum immunoprecipitation with H1 antiserum was found to occur with 3 min of formaldehyde treatment, which resulted in cross-linking of 10 to 20% of total H1 protein and immunoprecipitation of approximately 15 to 25% of ^{3}H-labeled DNA, with background immunoprecipitation less than 2% of the specific immunoprecipitation in the presence of 500 mM NaCl. The specificity of the H1 antiserum was demonstrated by immunoblot studies, examination of immunoprecipitated proteins, and competition with exogenous histone and high-mobility-group proteins (10a).

H1 binding is altered in a gene-specific manner in different stages of development. It could be argued that the changes in histone-DNA interactions occurring in the *Drosophila* heat shock genes, the model chosen by Solomon et al. (28) and Nacheva et al. (23) for their studies on histone-DNA associations, are representative of all genes in all organisms. However, the DNase I hypersensitivity of the 5′ ends of both active and inactive heat shock genes (37) and the high density of polymerase molecules on these genes in the active state (15) suggest that the chromatin structure of heat shock genes may be different from that of the majority of normally transcribed genes. With this in mind, we examined four genes (transcribed by two different polymerases) whose transcriptional activities vary in each of three normal developmental states of *T. thermophila*: the conjugation-specific genes *ngoA* and *cnjB* and the ribosomal DNA (rDNA) and actin genes. DNA from immunoprecipitated chromatin was probed with ^{32}P-labeled (13) cloned fragments from these genes. An example of the autoradiograms derived from the hybridization of immunoprecipitated and total DNA with the actin gene of *T. thermophila* is shown in Fig. 1. Ratios of the autoradiographic signals from these hybridizations with four gene sequences are shown in Fig. 2, in which the values represent the ratios of the amount of a particular sequence in the immunoprecipitated DNA compared with the starting DNA (bulk).

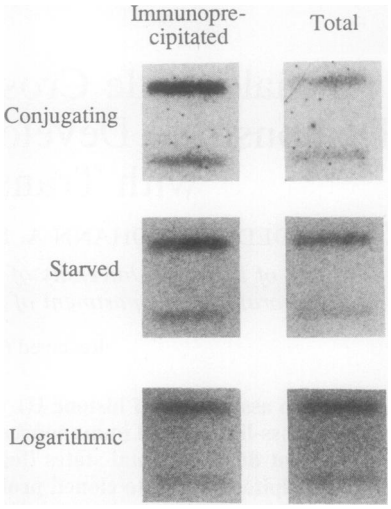

FIG. 1. Samples of slot blots of H1-immunoprecipitated and total DNA hybridized to an actin gene probe. Samples of 50 and 25 ng of immunoprecipitated or bulk (total) DNA were blotted onto Magnagraph (MSI), and the blots were hybridized (see reference 34 for conditions) with ^{32}P-labeled probes made from the *T. thermophila* actin gene (see legend to Fig. 2). Shown are the autoradiograms from the three different immunoprecipitation preparations.

For any particular probe, the immunoprecipitation can be compared between developmental states. The rDNA probe, for example, is relatively depleted in immunoprecipitated DNA (ratio of bound to total, <0.5) in log-phase chromatin

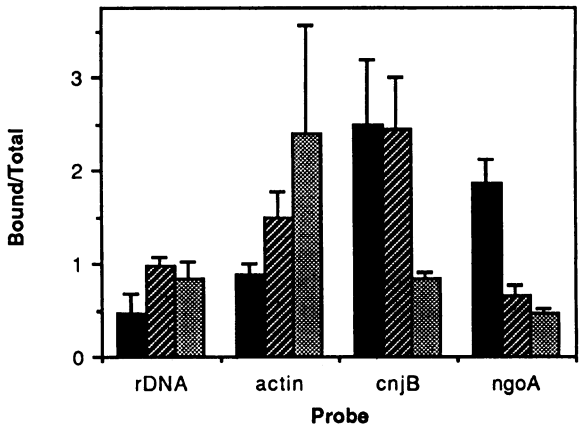

FIG. 2. Hybridization analysis of DNA immunoprecipitated with H1 antiserum. Formaldehyde-cross-linked solubilized chromatin was immunoprecipitated with anti-H1 antiserum, and the purified DNA was subjected to hybridization analysis. Blots were hybridized with ^{32}P-labeled probes made from the following cloned *Tetrahymena* gene fragments: pRP-9, a 2-kb *Hin*dIII fragment containing the 26S rRNA gene (rDNA;11); p583-2, a 970-bp *Bgl*II-*Hin*dIII fragment of the actin gene containing coding and 247 bp of 5′ noncoding DNA (8); and the conjugation genes pC2-1 and pC5-5, a 3.4-kb fragment of the *cnjB* gene and a 1-kb fragment of the *ngoA* gene, respectively (22). The autoradiographic signals were quantitated by densitometry. The data are presented as the ratio of the immunoprecipitated DNA signal (bound fraction) to the signal from bulk, unfractionated DNA (total). Error bars represent the range about the mean for two expriments. Solid bars, Logrithmically growing cells; cross-hatched bars, starved cells; stippled bars, conjugating cells.

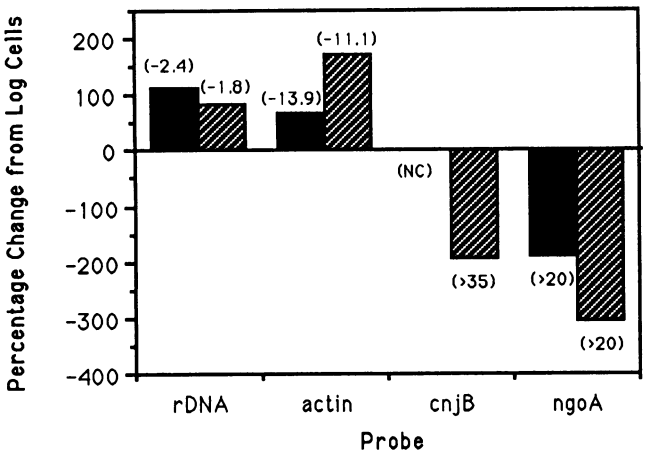

FIG. 3. H1 enrichment in immunoprecipitated DNA relative to logarithmically growing cells. Bound/total ratios (Fig. 2) from starved or conjugating cells relative to logarithmic cells were used to calculate the percentage change relative to the logarithmic state. The data thus represent the percentage increase or decrease of H1 cross-linking in starved (solid bars) or conjugating (cross-hatched bars) cells relative to logarithmically growing cells. Values in parentheses are the level of run-on transcription in starved and conjugating *Tetrahymena* cells relative to logarithmically growing cells from the data of Stargell et al. (28a) (see text).

and is increased in its representation in the bound fraction as the cell proceeds into starvation and subsequent conjugation. The conjugation gene *ngoA* shows the opposite effect; it is more enriched in the bound fraction in logarithmically growing cells than in starved or conjugating cells.

Figure 3 presents an arrangement of the data that facilitates comparison between genes as well as between states for a single gene. In this figure, the data are presented as the percentage increase or decrease in the enrichment of gene sequences in the H1 immunoprecipitate from starved and conjugating cells relative to the logarithmic state. Compared with log-phase cells, the immunoprecipitability of some genes (rDNA and actin) goes up in starved and conjugating cells while that of another gene (*ngoA*) goes down. The immunoprecipitability of the *cnjB* gene remains unchanged in starved cells but goes down considerably in conjugating cells. The important point to note is that immunoprecipitability with H1 antiserum is gene specific, not developmental stage specific. There is no simple tendency for all genes in a particular developmental stage to be associated with more or less H1 when analyzed by these techniques.

Changes in the immunoprecipitability of genes with anti-H1 serum are inversely correlated with transcriptional activity. Stargell et al. (28a) have analyzed the transcription rates of a variety of genes in nuclei isolated from growing, starved, or conjugating *T. thermophila*, and the ratios of the transcription rates for starved and conjugating cells relative to logarithmically growing cells have been incorporated into Fig. 3 (values in parentheses). As the transcription data show, the ribosomal and actin genes are transcribed at a lower rate in starved and in conjugating cells than in growing cells, while transcription of the *cnjB* and *ngoA* genes is detectable only in conjugating or starved and conjugating cells, respectively. Because some of the transcription rates are not detectable, we have presented these data as fold differences; thus, there is zero difference between two states in which transcripts are not detectable.

It is readily apparent for all genes analyzed that the run-on transcription data correlate inversely with the H1 immunoprecipitation findings (Fig. 3). Genes whose concentration increases in the immunoprecipitated chromatin of starved and conjugating cells (rDNA and actin) are also transcribed at a reduced rate relative to logarithmic cells. Likewise, the reduced presence of *ngoA* and *cnjB* in the immunoprecipitates from conjugating cells correlates with a marked increase in transcription of these genes in this state. Though the change in H1 immunoprecipitation between states is small, averaging about two- to three-fold, there is a significant correlation ($r^2 = 0.88$) between the level of change in transcription and the degree of change in H1 immunoprecipitation as the cells move from logarithmic growth into starvation or conjugation. The small changes in immunoprecipitation may be explained by the fact that there are multiple copies of each gene in the highly polyploid *Tetrahymena* macronuclear genome and all copies may not be transcriptionally active at any one time.

Changes in the physical association of H1 with DNA in different states of transcriptional activity. The simplest interpretation of our finding of an inverse correlation between the immunoprecipitability of specific genes with anti-H1 serum and their transcriptional activity is that there is a decrease in the amount of H1 associated with transcribed genes. This view would support previous findings that suggest an absence of H1 on active genes (9, 10, 26; reviewed in reference 31). However, consideration of the fixation-immunoprecipitation procedure and the nature of DNA-protein interactions allows other interpretations. It is possible that the H1-DNA contacts on active genes change in a way that renders the H1 unable to form a formaldehyde-mediated cross-link to DNA even though it remains associated with the active gene. Nacheva et al. (23) studied H1 cross-linking to a heat shock gene in *Drosophila* tissue culture cells by using two depurination-reduction cross-linking protocols. Their studies clearly demonstrated that the activation resulted in a loss of cross-linking via the globular core region, with little or no effect on cross-linking through the terminal regions. Thus, under their conditions, a transcription-associated change in the nature of the H1-DNA interaction occurs without complete removal of the protein from contact with the gene. While a similar phenomenon could explain the results reported here, a number of differences between our studies and those of Nacheva et al. are worth noting. *Tetrahymena* H1 does not possess the conserved central globular region typical of metazoan H1s. It does contain an unusually large number of evenly distributed basic residues (55 lysines and 3 arginines out of 173 amino acids), the primary amino acids cross-linked to DNA by formaldehyde (25, 35). Lysine-specific depurination-reduction and formaldehyde cross-linking should give similar results since they both require short distances (<0.2 nm) between the reactive groups (20, 23) and rely on the proximity of lysine to purines, in which exocyclic nitrogens serve as the second reactive site for formaldehyde (5). It seems likely that if any region of the H1 were still in close contact with the transcriptionally active DNA in *T. thermophila*, it would probably be cross-linked by formaldehyde.

Another possible explanation for the results reported here is that H1 molecules remain associated with active genes but somehow lose their antigenicity. It is unlikely that transcriptional activity causes a conformational change in H1 that results in reduced antibody reaction with epitopes, in light of recent findings of immunoreactive H1 associated with transcriptionally active regions of polytene chromosomes (12,

19). It is possible that the protein has reacted with formaldehyde to mask the epitope(s). If such an immunologic artifact is occurring in our studies, it is specific, reproducible, and correlated with changes in transcriptional activity. More importantly, it represents an altered H1 structure or H1-DNA interaction.

Supporting evidence for the presence of H1 on active genes comes from the work of Colavito-Shepanski and Gorovsky (7), who showed that the ratio of H1 to DNA (and to core histones) in isolated *Tetrahymena* chromatin highly enriched in active ribosomal genes was the same as that of total (nonribosomal) macronuclear chromatin. In the present experiments, ribosomal genes in a state of higher transcriptional activity are inefficiently immunoprecipitated with H1 antiserum (bound/total < 0.5; Fig. 2). These results argue that in growing *Tetrahymena* cells, H1 is still present on active ribosomal genes, but the association is changed so that the H1-DNA interaction is no longer sufficiently intimate to allow efficient cross-linking with formaldehyde, resulting in reduced immunoprecipitation. In this manner, behavior of the entire H1 molecule of *T. thermophila* would resemble that of the H1 globular core in transcriptionally active *Drosophila* heat shock genes. It seems likely that reduction of the H1-DNA interaction without complete dissociation of H1 is a general feature of transcriptional activation in eukaryotes. It remains to be determined whether this change precedes and is a prerequisite for transcription or is a consequence of passage of RNA polymerase molecules through an active gene.

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